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ARTÍCULO

Antitumoral activity of oncolytic vaccinia virus expressing the interferon-induced ds-RNA dependent protein kinase PKR

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ABSTRACT

Tumour cells generally become more susceptible to virus infection than normal cells due, in part, to a deficient interferon (IFN)-induced antiviral pathway. One of the key IFN-induced enzymes with potent antiviral action is the ds-RNA dependent protein kinase PKR, that once activated blocks protein synthesis, triggers apoptosis and prevents cell growth. Among viruses, vaccinia virus (VACV) lacking selected viral genes or armed with cytokines or tumour specific antigens has been used in preclinical and clinical studies as a therapeutic agent against different tumours. Here we showed in a mouse model of aggressive cancer by subcutaneous inoculation with prostate TRAMP-C1 cells, that a VACV recombinant expressing low levels of human PKR (VV-PKR) and lacking thymidine kinase (TK), is capable of reducing tumour burden when administered by a systemic route in immunocompetent C57/BL6 mice. In addition, expression of PKR was found to attenuate the virus, thus ensuring safety. A catalytically inactive enzyme PKR with a point mutation (K296R) induced similar oncolytic activity as the control virus lacking TK. These find-

ings suggest that VACV recombinants expressing PKR are candidate vectors against cancer cells.

Keywords: Protein kinase PKR; recombinant vaccinia virus; oncolytic activity; prostate tumours; mouse model.

RESUMEN

Actividad oncolítica del virus vaccinia que expresa la proteína quinasa PKR inducida por los interferones y dependiente de RNA bicatenario.

Las células tumorales son generalmente mas susceptibles a la infección viral que las células normales debido, en parte, a tener deficiencias en el sistema de señalización por los interferones (IFN). Uno de los enzimas clave inducidos por IFN es la proteína quinasa PKR dependiente de RNA bicatenario, que una vez activada fosforila el factor de iniciación eIF-2 alfa lo que conlleva a una inhibición generalizada en la síntesis de proteínas, inducción de la apoptosis y prevención del crecimiento celular. Como agente terapéutico frente a distintos tumores se está utilizando en preclínica y clínica el virus vaccinia que carece de ciertos genes virales, expresa ciertas citoquinas o antígenos tumorales. En esta investigación demostramos en un modelo agresivo de cáncer en ratón por inoculación ex vivo de células tumorales de próstata TRAMP-C1, que se reduce el crecimiento tumoral tras la infección a ratones inmunocompetentes C57/BL6 por vía sistémica de un virus recombinante de vaccinia que expresa bajos niveles de PKR. Además, la expresión de PKR conduce a la atenuación del virus en los tejidos. Un mutante catalíticamente inactivo de PKR produce niveles similares de actividad oncolítica, pero con replicación viral en tejidos. Estos resultados indican que vectores recombinants de vaccinia que expresan PKR son candidatos como vectores para luchar contra tumores.

Palabras Clave: Proteína quinasa PKR; virus vaccinia recombinante; actividad oncolítica; tumor de próstata; modelo ratón.

1. INTRODUCTION

Cancer is a multi-faceted process by which cells are sequentially transformed, lose cell growth control, develop tumours at specific sites and cells spread to different tissues leading to metastasis. Different strategies to fight cancer are used in the clinic, from surgery to chemotherapy, immune-therapy and more recently, although experimentally, through the use of viral vectors with the ability to destroy tumour cells. This therapeutic strategy uses oncolytic viral vectors with preference for replication in tumour cells, causing cell destruction (1). Vaccinia virus has a strong oncolytic effect due to its fast replication cycle and a high innate tropism to cancer tissue (2). Tumour targeting can be further improved by deleting vaccinia virus genes that are necessary for replication in normal cells but not in cancer cells. For example, deletions of either thymidine kinase (TK), vaccinia virus growth factor (VGF), or both have been shown to reduce pathogenicity compared to wild-type virus (3, 4). To enhance antitumor potency, oncolytic vaccinia viruses can be armed with therapeutic transgenes, such as immunostimulatory factors or suicide genes. The clinical data obtained thus far with the oncolytic poxvirus vectors shows that intratumoral injection and replication lead to reproducible delivery of the virus to systemic metastasis through the bloodstream with tumour necrosis; however, hurdles remain. Neutralizing response to the virus vector, nature of the inflammatory response required for tumour regression and limited application to locally advanced tumours, are among obstacles to be solved. Thus, development of poxvirus vectors with improved oncolytic characteristics are needed.

To search for novel viral vectors with enhanced oncolytic activity, we have taken advantage of a property of cancer cells, which frequently lose their ability to produce or respond to interferons (IFN). These molecules play critical roles in host defence against viral infections, control of cell growth and immune modulation (5). The poor response of tumour cells to IFN make these cells more prone to viral infection, hence forming the basis by which oncolytic viruses are used to destroy tumour cells as they replicate to a better extent in tumour cells than in normal cells. Among the proteins induced by IFN with antiviral importance is the ds-RNA dependent protein kinase PKR, an en-

zyme that when activated by dsRNA is autophosphorylated leading to phosphorylation of the alpha subunit of the eukaryotic initiation factor eIF-2 (eIF2 α) and, in turn, to inhibition of protein synthesis, induction of apoptosis and cell death (for review of PKR see (6)). Since PKR induction is dependent on sensitivity of the cells to IFN, we reasoned that a VACV vector expressing PKR should infect and destroy better cancer cells than normal cells, as the latter have a fully active IFN system. Thus, in this investigation we have explored the oncolytic ability of a VACV recombinant expressing low levels of PKR (referred to as VV-PKR) in cultured tumour cells and in a mouse model after subcutaneous inoculation of prostate tumour cells (TRAMP-C1). The use of a viral recombinant expressing low levels of PKR is because high levels of expression of PKR prevent virus growth (7, 8). Our findings showed that VV-PKR replicate in cultured tumour cells, but when inoculated in subcutaneous TRAMP-C1 tumour-bearing mice causes reduction of tumour burden in spite of restricted virus growth.

2. MATERIALS AND METHODS

2.1. Cells and viruses

TRAMP-C1 murine prostate cancer cell line obtained from American Type Culture Collection was maintained in DMEM with 10% foetal calf serum (FCS) and antibiotics. Cells reaching 95% confluence were shortly trypsinized, and harvested with serum-containing medium. The cells were washed and resuspended in serum-free complete medium at concentration of 5×10^6 viable cells in 1 ml. Half-million TRAMP-C1 cells in 100 μ l were implanted subcutaneously into the right flank. Tumour volume was estimated using the formula: $m_1^2 \times m_2 \times 0.5236$, where m_1 and m_2 represented the short and long diameter of the tumour, respectively. Tumours were measured using a digital calliper twice weekly until day 55 post-implantation or until the tumour burden has met the humane endpoint. Tumours that could not be measured with calliper but were found to infiltrate surrounding tissues at the necropsy were assigned as immeasurable. Tumour-free status was assigned to mice that at the necropsy have shown lack of residual tumour tissue at the implantation site.

The Western Reserve strain of vaccinia virus was used to generate the recombinant viruses expressing the WT and the human mutant PKR proteins. The recombinant genes were inserted in the TK locus of viral genome. PKR expression is regulated by the lac I repressor gene that is under the control of VACV early-late promoter p7.5. The mutant PKR, K296R has lost the catalytic activity of PKR due to substitution of the lysine 296 by arginine. The mutant VACV viruses expressing PKR (VV-PKR), its inactive mutant form (VV-K296R) and luciferase (VVLUC) have been previously described (9, 10). Viruses were grown in monkey BSC-40 cells and purified by sucrose gradients.

TRAMP-C1 cells grown in 12-well plates were infected at low 0.01 pfu/cell or high 5 pfu/cell virus multiplicities. Following virus adsorption for 60 min at 37 °C, the inoculum was removed and cells incubated with fresh DMEM containing 2% FCS at 37 °C in a 5% CO₂ atmosphere. At different times postinfection, cells were collected by scraping and used either for Western blot or virus titration. At 24 h postinfection (hpi), infected cells were lysed in Laemmli buffer, cell extracts fractionated by 12% SDS-PAGE and analyzed by Western blot using rabbit polyclonal anti-vaccinia serum, anti-PKR or anti-phospho PKR. Virus yields were determined by plaque formation at 48 hpi in monkey BSC-40 cells after staining with 1% crystal violet in 2% ethanol.

2.2. Evaluation of virus pathogenicity

Mice C57/BL6, four per group, were inoculated at days 0 and 3 with a dose of 5×10^7 pfu per mouse with different viral recombinants, VVLUC, VV-K296R or VV-PKR, by either intraperitoneal or intranasal routes. Mice were weighed daily and signs of illness were scored. Virus was titrated from homogenates of spleen and lung of each mouse at different days post-infection by a plaque assay in monkey BSC-40 cells.

2.3. Evaluation of oncolytic activity

Male mice C57/BL6 seven weeks old, 10 animals per group, were injected by subcutaneous route with 1×10^6 of TRAMP-C1 of mouse prostate cancer cells. At the median tumour volume of 75 to 100 mm³

(30 days post-inoculation), mice were injected by intraperitoneal route with 5×10^7 pfu of vaccinia virus VVPKR, VVLUC, VVK296R, or saline PBS. Virus inoculation by i.p was performed twice in animals with developed tumours, giving the virus at times 0 and after 3 days. Tumours were measured every 3-4 days using digital calliper.

3. RESULTS

3.1. VACV recombinants with PKR under control of the lac I operator/repressor system induced PKR in tumour prostate cancer cells

We have previously described a VACV recombinant expressing PKR (VV-PKR) under control of the inducible lac I operator/repressor system (9, 11); scheme in Figure 1A). Briefly, when cultured cells are infected with VV-PKR in the presence but not in the absence of IPTG, PKR is produced at high levels and this leads to its own phosphorylation triggered by the viral dsRNA produced during infection from symmetrical transcription, followed by phosphorylation of eIF-2 α and in turn, inhibition of protein synthesis, activation of caspases, induction of apoptosis and cell death (6, 12). However, we noted that when tumour cells TRAMP-C1 are infected with VV-PKR in the absence of IPTG there low levels of PKR are produced when compared to the infection in the presence of IPTG (Figure 1B). Similar observation was found when TRAMP-C1 cells were infected with the mutant form of PKR (K296R), in which case the presence of IPTG markedly increased the levels of PKR (Figure 1B). As expected, high levels of PKR produced in the presence of IPTG correlated with phosphorylation of eIF-2 α , while low levels of PKR in the absence of IPTG induced lower eIF2 α phosphorylation. This was not observed when the catalitically inactive mutant form of PKR (K296R) was synthesized (Figure 1B). As determined by Western blot with antibodies against vaccinia proteins, the accumulation of viral proteins was not impaired in TRAMP-C1 cells infected with VV-PKR in the absence of IPTG, while it was blocked when IPTG was present (Figure 1C). Moreover, virus yields were similar in cells infected with the control virus VV-LUC, a virus expressing luciferase marker in the TK locus, as in cells infected with VV-PKR in the absence of IPTG (Figure 1D),

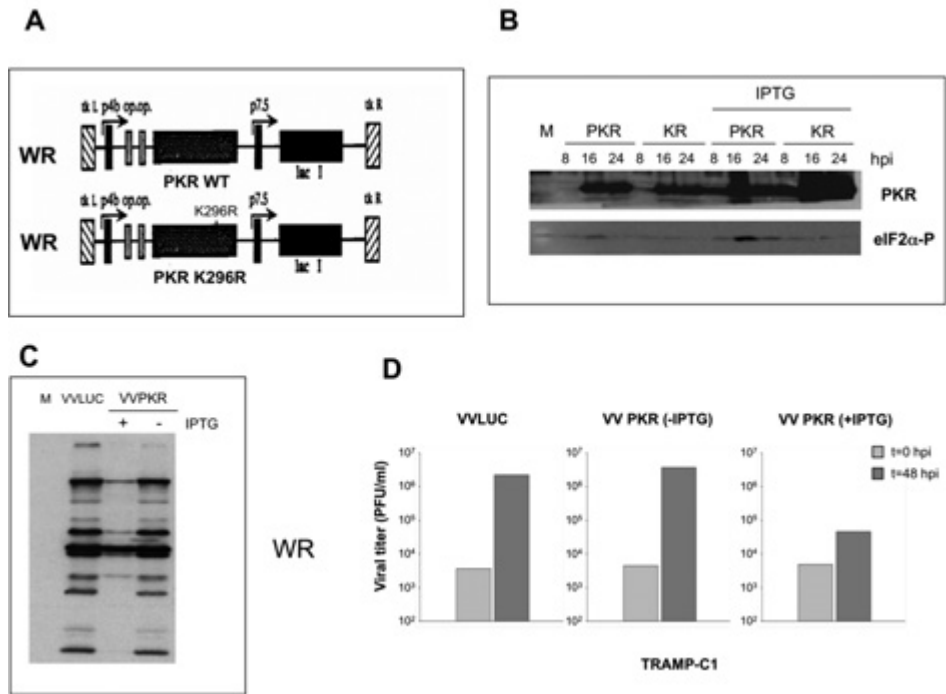


Figure 1. Characteristics of vaccinia virus recombinant vectors expressing PKR.
A. Scheme of the recombinant vaccinia virus vectors. The Western Reserve strain of vaccinia virus was used to generate the recombinant viruses expressing the human wild type (WT) and the mutant PKR proteins. The recombinant genes were inserted in the TK locus of viral genome. PKR expression is regulated by the lac I repressor gene that is under the control of VV early-late promoter p7.5. The mutant PKR, K296R has lost the catalytic activity of PKR due to substitution of the lysine 296 by arginine. **B.** TRAMP-C1 cells were infected with 5 pfu/cell of vaccinia virus recombinants VV-PKR and VV-K296R in presence or absence of the inducer IPTG (5 mM). The PKR expression and PKR catalytic activity was analyzed at different time post-infection using specific antibodies against total PKR and the phosphorylated form of eIF2α. **C.** TRAMP-C1 cells were infected with 5 pfu/cell of vaccinia virus recombinants VVLUC and VV-PKR in presence or absence of the inducer IPTG (5 mM). The vaccinia virus proteins at 24 hpi were detected using specific polyclonal antibody to VACV proteins. **D.** TRAMP-C1 cells were infected with 0.001 pfu/cell of vaccinia virus recombinants VVLUC and VVPR in presence or absence of the inducer IPTG (5 mM). Virus yields were measured at 0 and 48 hpi after titration by plaque assay in monkey BSC-40 cells.

while in the presence of IPTG virus yields were inhibited. Induction of PKR in TRAMP-C1 cells infected with VV-PKR could be due to leakiness of the promoter or as a result of the viral infection.

The above findings establish that VV-PKR can be used as a vector to produce low levels of PKR in tumour TRAMP-C1 cells.

3.2. Attenuation of VV-PKR in infected mice

To evaluate whether low level expression of PKR could be biologically active leading to virus attenuation, we performed pathogenicity studies in mice, by measuring survival, virus titration in tissues and humoral immune responses to the virus. Thus C57/BL6 mice, 4 animals per group, were injected either by intraperitoneal (i.p.) or intranasal (i.n.) routes with 5×10^7 plaque forming units (pfu) of either VV-LUC, VV-PKR and VV-PKR(K296R) or with a solution of PBS. Several signs of viral illness, like ruffled fur, rigidity, lack of activity and mortality were evaluated with time of infection. As shown in Figure 2A, i.n. inoculation of VV-LUC results in severe illness and the animals had to be sacrificed by day 6 due to the loss of 30% of their body weight, while animals that received VV-PKR recovered the weight loss by day 7 after viral infection. Animals inoculated with VV-PKR(K296R) behaved similarly as VV-LUC (not shown). The signs of illness correlated well with virus abundance in lung tissues, with about 5 log reduction at day 3 in mice inoculated i.n. with VV-PKR in comparison with VV-Luc (Figure 2A, lower panel). When animals were inoculated with the different viruses by i.p. routes, VV-Luc or VV-PKR(K296R) induced weight loss while infection with VV-PKR did not (Figure 2B); this correlated with lower virus titers in the spleen compared with animals administered with VV-LUC or VV-PKR(K296R) (Figure 2B, lower panel). To provide further support for reduced replication of VV-PKR in mice, we evaluated by ELISA the antibody response against the viral recombinants at 30 days after i.p. inoculation with the different viruses. As expected, VV-PKR induced lower antibodies against VACV antigens than either VV-LUC or VV-PKR(K296R) (Figure 3). The findings of Figures 2 and 3 establish that VV-PKR is attenuated in mice when inoculated by i.n. or i.p. routes and induces low levels of antibodies against the virus vector.

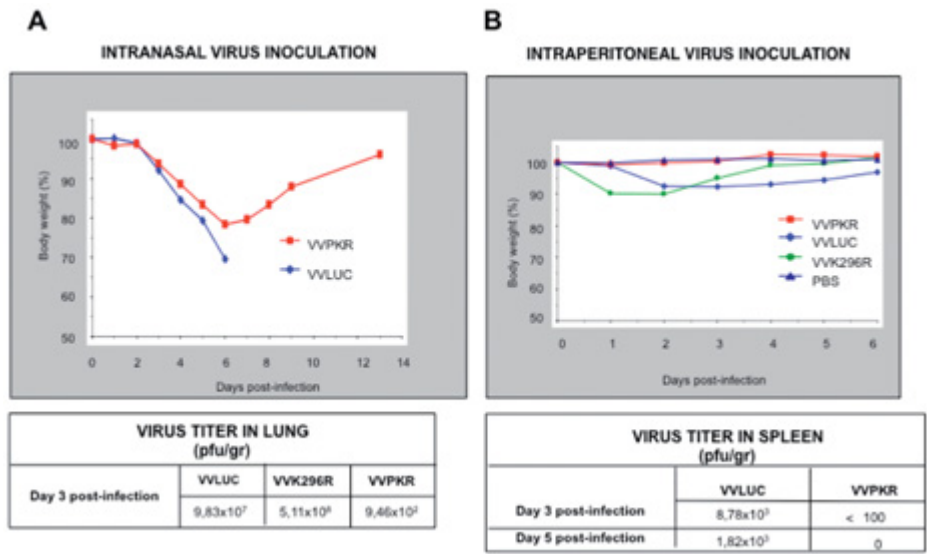


Figure 2. VV-PKR shows reduced pathogenicity in comparison to VV-LUC and VV-K296R in C57/BL6 mice. **A.** Mice C57/BL6, four per group, were inoculated with 5×10^7 pfu per mouse of VV-LUC or VV-PKR by intranasal route. Mice were weighed daily. Virus was titrated in lungs at third day post-infection. **B.** Mice C57/BL6, four per group, were inoculated with 5×10^7 pfu per mouse with VV-LUC, VV-K296R, VV-PKR or PBS by intraperitoneal route at days 0 and 3. Mice were weighed daily. Virus was titrated in spleen at third and fifth days post-infection. Mean values titers are shown.

3.3. Systemic inoculation of VV-PKR in mice with transplantable prostate cancer cells reduces tumour burden

Next we wanted to define if VV-PKR could be used as an oncolytic vector. Thus, C57/BL6 mice were inoculated by subcutaneous route with TRAMP-C1 prostate tumour cells and when the tumour median volume reached 75 to 100 mm³, 10 mice per group were injected by i.p. route with 5×10^7 pfu of different VACV vectors: VV-LUC, VV-PKR and its mutant VV-PKR(K296R), or control PBS. Virus was inoculated twice with the same dose, three days apart. Tumour size was recorded every 3-4 days using a digital caliper. As shown in Figure 4, the tumour size was reduced in mice inoculated with the three viral

vectors, although there was a trend of slightly higher tumour reduction in mice inoculated with the viral vectors expressing PKR. The virus titers at the tumour site remain elevated in animals inoculated with VV-Luc compared to animals inoculated with VV-PKR. At day 5 after the administration of two doses of virus by i.p. inoculation, the mean virus titers in 3 mice were 3.9×10^6 pfu/gr tissue in the tumours of VV-LUC compared to less than 10^2 in VV-PKR.

Previously, it has been shown that virus lacking TK improves the oncolytic activity of VACV (3). Our results showing that VV-PKR exhibits reduced replication in tissues compared to either VV-LUC or VV-PKR(K296R), together with the fact that tumours were reduced similarly by VV-PKR as for the other two vectors, indicate that VV-PKR is a self-limited replication vector with oncolytic activity.

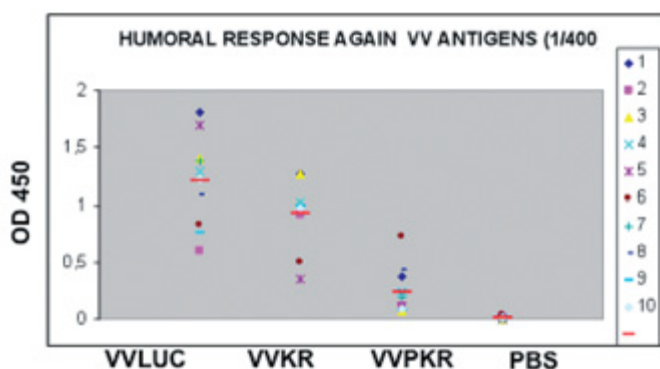


Figure 3. Antibody response against different VACV recombinants. Detection of antibodies against vaccinia virus (by ELISA) in serum obtained 23 days post-infection from mice, four per group, inoculated i.p. twice (days 0 and 3) with 5×10^7 pfu per mouse of each recombinant viruses VVLUC, VV-PKR, VV-K296R or PBS. 96-well plates were fixed with $1 \mu\text{g}/\text{well}$ of soluble cell extract from VACV-infected BSC-40 cells. A single serum dilution of 1:400 was used for all samples. Absorbance of serum dilution from each mouse is shown.

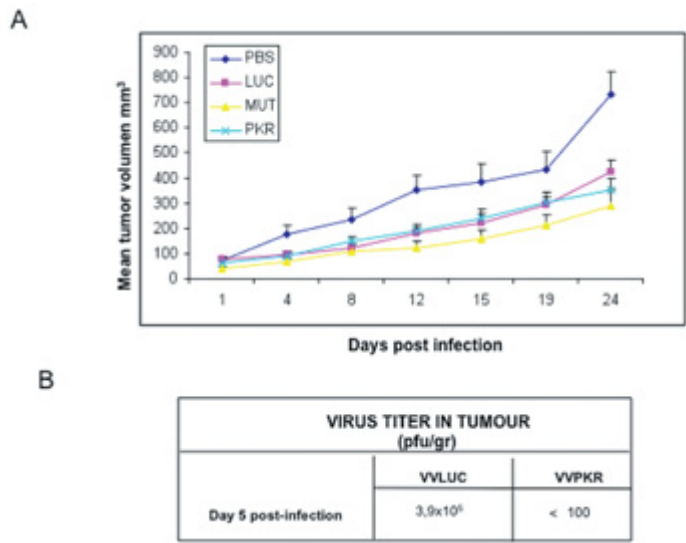


Figure 4. Diminished TRAMP-C1 tumour growth in C57/BL6 mice treated with recombinant vaccinia viruses VV-PKR, VV-K296R and VVLUC. A. Male mice C57/BL6 seven weeks old, 10 per group, were injected by subcutaneous route with 1×10^7 of TRAMP-C1 mouse prostate cancer cells. At the median tumour volume of 75 to 100 mm³ (30 days post-inoculation), mice were injected by intraperitoneal route with 5×10^7 pfu of vaccinia virus VVPCR, VVLUC, VVK296R, or saline PBS on days 0 and 3. Tumours were measured every 3-4 days using digital caliper. **B.** Virus titers in transplantable tumours. Shown are the mean virus titers in pfu/gr of tumour tissue obtained by surgery 5 days after the second dose of virus was administered by i.p. route.

4. DISCUSSION

Oncolytic vaccinia viruses have demonstrated tumour specificity, high levels of transgene expression, and anti-tumour effect (13-15). PKR is an interferon-induced kinase that is able to induce apoptosis through eIF2 α and NFkB modulation, and have a discussed tumour suppressor activity. PKR is also involved in the pathways of several tumour suppressors such as p53, IRF1, ARF and MDA7 (6). To define the contribution of PKR as an oncolytic agent, we have evaluated the antitumoral activity of a recombinant vaccinia virus expressing PKR inserted in the TK locus, in immunocompetent C57/BL6 mice bearing subcutaneous tumours produced by the prostate cancer cell line

TRAMP-C1. We found that VV-PKR replicates efficiently in prostate tumour TRAMP-C1 cultured cells while it expresses low levels of PKR. However, inoculation of VV-PKR in mice by either i.n. or i.p. routes results in reduced virus replication in tissues and animals recovered from viral infection. Significantly, the establishment of an aggressive tumour in mice by subcutaneous inoculation of TRAMP-C1 cells was markedly reduced following systemic inoculation with VV-PKR. This reduction in tumour burden was similar to that induced by viral vectors lacking TK, similar to VV-LUC or the catalytically inactive mutant VV-PKR(K296R). Since VV-PKR also lacks TK but has a reduced capacity to propagate in tissues than either VV-LUC or VV-PKR(K296R), the fact that the vector inhibits the tumour growth similarly as the two other vectors, suggest that the contribution of PKR is important to restrict tumour growth. This could be mediated by activation of PKR in infected tumour cells leading to apoptosis and cell death, while neither VV-LUC or VV-PKR(K296R) are able to induce apoptosis (16, 17). However, we could not detect virus propagation in most of the animals inoculated with VV-PKR at the tumour site, while virus was easily found in tumours from animals inoculated by i.p. with VV-LUC or VV-K296R. This indicates that factors other than virus replication in tumour cells are responsible for PKR-induced tumour reduction. The reduced virus propagation of VV-PKR will have the added advantage that antibody responses against the vector will be minimized. In fact, we observed that in mice inoculated i.p. with VV-PKR the levels of antibodies produced against the virus vector are about 5-fold lower than those triggered by either VV-LUC or VV-PKR (296R). This characteristic made it possible the administration in vivo of repetitive doses of VV-PKR at the tumour site or systemically as shown here, while a fully replicating VACV vector triggering strong antibody response against itself will be more restricted after additional doses of the vector are inoculated.

Why PKR may be an advantage as an inhibitor of tumour growth? Considering its mode of action, PKR has been shown to act in concert with major inflammatory kinases and directly interact with a critical insulin signalling component, suggesting PKR as a core component of a putative metabolic inflammasome that consist of major elements in inflammatory signalling and insulin action, which can represent a central mechanism for the integration of pathogen response and innate

immunity (6, 18). Based on previous observations on the mode of action of PKR and our findings here in mice, we suggest that VV-PKR might exert its antitumour action not by direct virus replication at the tumour site but through the triggering of innate immune pathways. In fact, PKR has been shown to activate multiple signalling pathways (6). By microarray analyses in human tumour cells infected with VV-PKR we have shown that expression of PKR triggered the induction of 111 genes, of which 97 were upregulated, among those the ATF-3 transcription factor involved in stress-induced B-cell apoptosis, which might also contribute to the antitumor activity of PKR (19). Recently, it has been shown that induction of the tumour suppressor p53 by DNA-damaging stress results in significant increase in the expression of PKR and in turn in PKR-associated biological functions like a translational block and apoptosis, thus contributing to tumour suppression (20). Undoubtedly, induction of PKR in the context of a tumour cell together with its activation by the dsRNA provided from the vector VV-PKR has the advantage that it provides the signals that lead rapidly to innate immune cell activation, like the inflammatory complex, and in turn to tumour growth control.

The oncolytic VACV vectors that have been developed to date are either lacking one or several viral genes or expressing exogenous products that improve the killing ability of the virus vector. Among deleted viral genes: TK (thymidine kinase) which produces a nucleotide pool for replication of the viral genomes, vaccinia growth factor (VGF) that activates the epidermal growth factor receptor, and a variety of immunosuppressive proteins, like B18R which binds and sequesters type I interferons (4). Improvements in the killing activity of the vector are a variety of molecules, like vectors expressing cytokines, anti-angiogenic agents, agents that disrupt the extracellular matrix to improve viral spread, and prodrug-converting enzymes (21-23). Phase I and II clinical trials are ongoing with some of the oncolytic VACV vectors. The most advanced vector in the clinic is the one that had inactivated both TK and VGF and also expresses GM-CSF (21). While all of the oncolytic VACV vectors developed had full replication capacity, which makes them more vulnerable to attack by CTLs and antibodies, the vector VV-PKR described here has more restricted replication, but importantly triggers low antibody response against itself and reduces tumour burden.

Overall, our studies provided evidence for a new class of an oncolytic VACV vector inducing low levels of PKR in tumour cells and lacking TK, as a product to selectively destroy tumour cells, not by direct killing but probably by the triggering of innate immune mechanisms, like the inflammasome.

5. ACKNOWLEDGEMENTS

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